Respiratory Medicine (2000) **94,** 10–17
Article No. rmed.1999.0700

Regulated exocytosis in immune function: are SNARE-proteins involved?

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Inflammation is an important feature in the pathogenesis of most chronic lung diseases. It is characterized by tissue infiltration with various inflammatory cells, including eosinophils, mast cells, basophils, macrophages, neutrophils, T- and B-lymphocytes and dendritic cells (1). In the tissue granulocytes release their toxic granule proteins after being stimulated by soluble mediators released by other inflammatory cells (2). Therefore, it is important to characterize the intracellular mechanisms regulating the transport of the granule contents in inflammatory cells.

Intracellular vesicle-traffic in mammalian cells is mediated by transport vesicles that emerge from donor compartments and are specifically targeted to acceptor compartments where they deliver their contents after membrane fusion (3). This traffic leads to three types of fusion results: vesicle–intracellular membranes, vesicle–vesicle or vesicle–plasma membrane. The process leading to fusion of vesicle–plasma membrane is called exocytosis, and it delivers proteins to the cell surface (receptors e.g. CD11b, CD18) and exports soluble molecules (mediators e.g. eosinophil cationic protein ECP) from the cell. A number of key proteins involved in regulated exocytosis have been identified from inflammatory cells. This review is a brief summary of these proteins and it includes recent results from studies on regulated exocytosis in inflammatory cells.

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Cellular secretory pathways

Protein secretion from mammalian cells can be either constitutive or regulated (4,5) (Table 1). While both pathways exist in most cells, the stimuli required for, and products released by, regulated exocytosis are specific for differentiated cells.

The SNARE-hypothesis

Regulated exocytosis occurs through a pathway consisting of two steps: Interaction of vesicles with the plasma membrane (docking) and Ca^{2+}-triggered membrane fusion [Fig. 1(a)].

Most of the proteins participating in regulated exocytosis were first biochemically characterized and cloned from neurons (6,7). The physical contact between the synaptic vesicles and the plasma membrane is thought to be mediated by specific protein–protein interaction. The SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor)-hypothesis attempts to explain this interaction (8,9). The original SNARE-hypothesis postulates that vesicular trafficking in a mammalian cell involves a unique vesicle-bound ligand (v-SNARE) that specifically recognizes and interacts with two unique receptor target molecules (t-SNAREs) found in the plasma membrane. In synapses a vesicle associated membrane protein called VAMP-1 (or synaptobrevin-1) has been identified as a v-SNARE, which interacts with the t-SNAREs syntaxin-1 and SNAP-25 (synaptosomal-associated protein of 25 kDa) in the plasma membrane. These three proteins form the SNARE-complex.

The essential role of these SNARE-proteins in regulated exocytosis has been established by their sensitivity to specific proteolytic cleavage by the clostridial neurotoxins Tetanus toxin and Botulin toxin (10).

Synaptotagmin, a vesicular integral membrane protein, interacts transiently with the SNARE-complex via SNAP-25 (11). According to the hypothesis an increase of intracellular Ca^{2+}-concentration triggers the association of the cytosolic proteins α- and γ-SNAP (soluble NSF attachment protein, with no homology to SNAP-25) to the complex by displacing synaptotagmin followed by

Received 18 August 1999 and accepted 1 September 1999.
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0954-6111/00/010010+08 $35·00/0

TABLE 1. Presentation of regulated and constitutive secretory pathway

Secretory pathway	Outer signal requirement	Secretory form	Accumulation of secretory granules	Protein content in secretory granules
Regulated pathway	Yes	Rapid	Yes	High
Constitutive pathway	No	Constitutive	No	Low

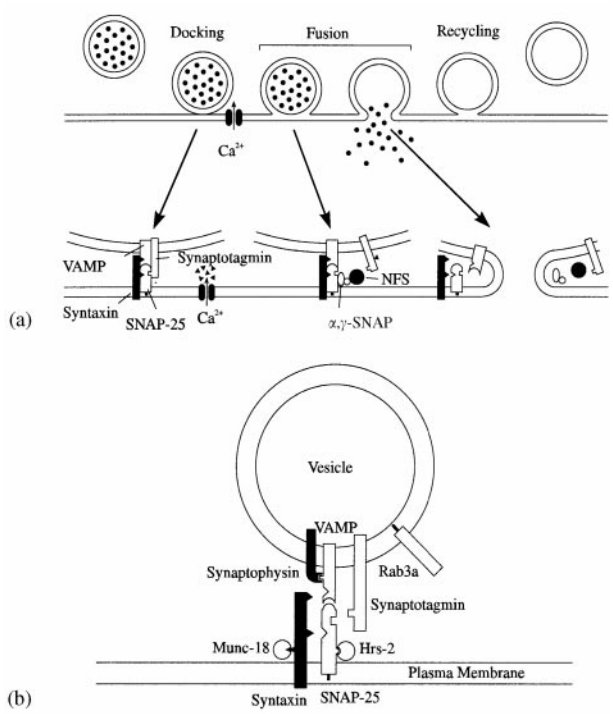

FIG. 1. (a) Presentation of the order of events leading to vesicle–plasma membrane fusion in the SNARE-hypothesis. In the docking step a SNARE-complex consisting of VAMP, syntaxin and SNAP-25 is formed. Synaptotagmin binds to the complex and is believed to work as a Ca^{2+}-sensitive clamp. Upon calcium-binding synaptotagmin dissociates from the SNARE-complex and allows α- and γ-SNAP to bind to the complex. The SNAPs recruit the ATPase NSF which hydrolyses ATP and thereby dissociates the SNARE-complex. This dissociation leads to fusion between vesicle and plasma membrane in which the vesicles release their content. Following the fusion step the vesicles are recycled. (b) Schematic presentation showing the localization and potential protein–protein interactions of the core proteins involved in regulated exocytosis.

recruitment of the ATPase N-ethylmaleimide-sensitive factor (NSF). Hydrolysis of ATP by NSF results in disruption of some SNARE-complex protein interactions before membrane fusion (12,13) and subsequently the vesicles are recycled (14).

Presentation of vesicle targeting receptors and proteins participating in regulated exocytosis

The range of the minimal set of proteins that the SNARE hypothesis identifies as central to the exocytosis is summarized in Table 2 and Fig. 1(b).

SYNTAXIN-FAMILY

The syntaxin-family includes at least 16 mammalian members and among these syntaxins 1A-B, 2, 3A-E and 4 participate in exocytosis (15,16). The syntaxin 1A and 1B are neuron-specific, while the other syntaxins are present in various tissues. They are integral membrane proteins with a transmembrane domain at the C-terminal that anchors the protein in the membrane. The cytoplasmic part has coiled-coil regions which interact with VAMPs and SNAP-25 (16).

SNAP-25

SNAP-25 exists in two isoforms, SNAP-25A and SNAP-25B, that arise by alternative splicing (17). It is mainly found in neuronal cells, where it is associated with the plasma membrane via plamitoylation on cysteine residues at the centre of the protein (18). Both the N-and C-terminals are located in the cytoplasm and contain coiled-coil regions which are believed to interact with syntaxins, VAMPs, synaptotagmin and SNAPs (19). SNAP-25 has an ubiquitously tissue-expressed homologue, which is called SNAP-23 (20). It is 59% identical to SNAP-25 and can, like SNAP-25, interact with both syntaxins and VAMPs.

VAMP (SYNAPTOBREVIN)-FAMILY

VAMP (synaptobrevin)-family currently has eight described members: VAMP-1–8 (12,21). VAMP-1 and VAMP-2 mainly exist in the brain, whereas the other VAMP-isoforms are ubiquitously expressed. VAMPs are integral membrane proteins being anchored to the membrane of secretory granules by a transmembrane region adjacent to the C-terminus. The conserved central domain is proposed to interact with syntaxins and SNAP-25 (22).

TABLE 2. Properties of mammalian proteins participating in regulated exocytosis

Protein prototype	Molecular weight	Subcellular localization	Putative function	References
Syntaxin	35 kDa	Plasma-/vesicle membrane (integral membrane protein)	t-SNARE	(15,56,61)
SNAP-25	25 kDa	Plasma-/vesicle membrane (palmitoylated to membrane)	t-SNARE	(18,56,62)
SNAP-23	23 kDa	Plasma membrane (palmitoylated to membrane)	t-SNARE	(20,63)
VAMP	18 kDa	Vesicle membrane (integral membrane protein)	v-SNARE	(12,64–66)
NSF	75 kDa	Cytoplasm	Disrupts the SNARE-complex	(24)
α-, γ-SNAP	35-, 39-kDa	Cytoplasm	Recruit NSF to the SNARE-complex	(67)
Synaptotagmin	65 kDa	Vesicle membrane (integral membrane protein)	Ca^{2+}-sensitive clamp	(25,68)
Rab3	25 kDa	Vesicle membrane (attached to membrane)	Regulator	(28)
Munc-18	67 kDa	Plasma membrane (attached to syntaxin)	Molecular clamp	(36)
Hrs-2	115 kDa	Plasma membrane (attached to SNAP-25)	Molecular clamp	(34)
Synaptophysin	38 kDa	Vesicle membrane (integral membrane protein interacting with VAMP)	Molecular clamp	(69,70)

For abbrevations see text.

NSF/SNAP-COMPLEX

The NSF/SNAP-complex is built of the ATPase NSF and the two SNAP-isoforms α- and γ-SNAPs (23). Both NSF and the SNAP-proteins are cytosolic proteins that are ubiquitously expressed. They are required in most intracellular vesicular fusion steps. The complex does not exist freely in the cytoplasm, but is formed upon binding of the SNAP-proteins to the SNARE-complex. This binding recruits NSF to the SNARE-complex, which hydrolyses ATP resulting in disruption of the SNARE-complex (24).

SYNAPTOTAGMIN

Synaptotagmin has at least a dozen mammalian isoforms, which all are located on vesicles (25). Synaptotagmin I and II are only expressed in the brain, whereas synaptotagmin III–XII are detected both in and outside the brain (26). All synaptotagmins have a N-terminal domain, which is inserted in the vesicular membrane, and a large cytoplasmic region with two Ca^{2+}-binding domains (27). Due to the ability of synaptotagmin to bind calcium, it is believed to trigger exocytosis. According to this highly debated postulate, with low intracellular Ca^{2+}-concentration synaptotagmin binds to the SNARE-complex and thus works as a clamp. But upon an outer Ca^{2+}-signal leading to an increase in intracellular Ca^{2+}-concentration, synaptotagmin binds calcium and is displaced from the SNARE-complex, thereby allowing binding of SNAP-proteins to the SNARE-complex (25).

RAB3 PROTEINS

The rab3 proteins consisting of rab 3A-D proteins are involved in regulated exocytosis (28). Rab proteins belong to a large GTP-binding protein superfamily, which exhibits low sequence identity except for GTP-binding regions central in the proteins (29). They participate in many steps of intracellular vesicle-trafficking (30).

The four rab3 isoforms are associated with secretory vesicles in a variety of cell types and differential expression of rab3 isoforms has been reported (30,31). They are associated with the vesicle membrane via geranylation on C-terminal cysteine residues. The exact role of rab3 proteins is unknown, but it is assumed that they regulate the assembly or fidelity of the SNARE proteins, for example rab3A may have two different roles: it efficiently docks vesicles to the plasma membrane (32) and, secondly, it regulates the efficiency of the fusion process (33).

HRS-2, SYNAPTOPHYSIN AND MUNC-18

Hrs-2, synaptophysin and Munc-18 bind to SNAP-25, VAMP and syntaxin, respectively (34–36). These three SNARE-interacting proteins may act as molecular clamps that inhibit vesicle docking until all elements of the

SNARE-complex are in place. It has been shown that the binding of Hrs-2 to SNAP-25 is inhibited by calcium in the concentration range that supports exocytosis (34). Hrs-2 may therefore prevent SNAP-25 from interacting with the other SNARE-proteins until the cell receives an outer signal, that results in an increase of intracellular Ca^{2+}-concentration. Hydrolysis of GTP by rab proteins is expected to cause the release of synaptophysis and Munc-18 from VAMP and syntaxin, respectively, which allows the v- and t-SNAREs to interact in the fusion reaction.

SNARE-proteins identified in inflammatory cells

NEUTROPHILS

Neutrophils play an important role in defence against infections and in the inflammatory process. They have four distinct cytoplasmic granules; primary, secondary, tertiary and secretory granules (37). Upon detection of bacterial invaders, neutrophils migrate to the infection-site where they endocytose foreign particles by phagocytosis. Neutrophils require cell adhesion molecules on the surface for diapedesis and migration through tissue. Surface translocation of secretory vesicles containing adhesion molecules is regulated by SNARE-complexes (38).

Several SNARE-proteins have been detected in neutrophils. Immunoblotting experiments have detected syntaxin-4, VAMP-2 and SNAP-25 (39,40) (Table 3). These proteins were present in the plasma membrane and secretory vesicles. Stimulation of neutrophils induced migration of VAMP-2 toward the plasma membrane (39). Two isoforms of SNAP-23 have been detected at the mRNA-level in neutrophils (41). The localization of these isoforms still has to be determined in these cells. Using reverse-transcription polymerase chain reaction (RT-PCR) we have not been able to detect SNAP-25 in neutrophils. Since RT-PCR is more sensitive than immunoblotting, the presence of SNAP-25 in neutrophils is questioned. The fact that SNAP-25 still has been detected in neutrophils by immunoblotting may be due to use of SNAP-25 antibodies that crossreact with SNAP-23.

MAST CELLS

Mast cells are highly specialized secretory cells that release pro-inflammatory mediators such as chemotactic factors and immunoregulatory cytokines (42). They contain large populations of secretory granules in their cytoplasm and undergo exocytosis after stimulation, often within few minutes (43), implicating their use of regulated exocytosis.

Syntaxin-3, syntaxin-4, VAMP-2 and SNAP-23 have been identified in mast cells by immunoblotting (44) (Table 3). The distribution of these four proteins has been confirmed biochemically (cell fractionation) and morphologically (immunofluorescence microscopy). SNAP-23 and syntaxin-4 are both plasma membrane proteins, while syntaxin-3 and VAMP-2 are detected as granular proteins. Immunofluorescent studies have indicated that SNAP-23 is relocated from the plasma membrane into the cell interior in stimulated cells. This relocalization is essential for the exocytosis as inhibition of this process delays exocytosis (44). Besides the granule–plasma membrane fusion of regulated exocytosis, mast cells can also induce fusion between two intracellular granules. The relocated SNAP-23 may therefore enable formation of SNARE-complexes between individual granules. SNAP-25 has not been detected in mast cells by immunoblotting (44).

By immunoblotting, immunofluorescence microscopy and cell fractionation, synaptotagmin-I has been localized to secretory granules (45) (Table 3). Synaptotagmin-I may have a function in exocytosis from mast cells, since transfection of mast cells with the synaptotagmin-I cDNA influenced the Ca^{2+}-dependent exocytosis (45).

TABLE 3. Presence of SNARE-proteins in inflammatory cells

Protein prototype	Neutrophils	Mast cells	Macrophages	Eosinophils	Basophils
Syntaxin	Syntaxin-3* Syntaxin-4	Syntaxin-3 Syntaxin-4	Syntaxin-2 Syntaxin-3 Syntaxin-4	Syntaxin-3*	?
SNAP-25	÷*	÷	?	÷*	÷
SNAP-23	+	+	?	+*	+*
VAMP	VAMP-1* VAMP-2*	VAMP-2	VAMP-related	VAMP-1* VAMP-2*	?
NSF	÷	?	+	?	?
α-, γ-SNAP	?	?	α-SNAP	?	?
Synaptotagmin	÷*	+	?	÷*	?
References	(39,41)	(44,45)	(47–49)	(52)	

Explanation of symbols: (÷) the protein or its mRNA not detected; (+) the protein or its mRNA detected; (?) no available data; * own unpublished resulting using RT-PCR on human cells. For abbreviations see text.

MACROPHAGES

Macrophages play a primary role in immune defence by phagocytosis of pathogens. During phagocytosis a phagosome is formed, which matures by specific fusion with intracellular vesicles. In addition to phagocytosis the macrophages also have a proinflammatory function by rapidly releasing cytokines (46).

Syntaxin-2, syntaxin-3 and syntaxin-4 have been identified in both the phagosomal and plasma membranes by immunoblotting (47) (Table 3). VAMP has also been detected in macrophages by immunoblotting (48) (Table 3). This protein was identified due to its recognition of VAMP antibodies in immunoblotting experiments and its sensitivity to Tetanus toxin. In addition NSF and α-SNAP have been identified on phagosomes containing a living intracellular pathogen *Listeria* (49) (Table 3). This implicates that SNARE complex proteins participate in endocytosis in macrophages as well.

EOSINOPHILS AND BASOPHILS

Both eosinophils and basophils release toxic granule proteins by degranulation, which plays a central role in host defence and in the pathophysiology of diseases associated with eosinophils and basophils. The degranulation process in both eosinophils and basophils is believed to happen through regulated exocytosis, since the process is very rapid (50,51). By RT-PCR we have identified syntaxin-3A, VAMP-1, VAMP-2 and SNAP-23 in human eosinophils, whereas SNAP-25 and synaptotagmin were not expressed in these cells (Table 3). In addition, functional experiments showed that pretreatment of eosinophils with Tetanus toxin, which cleaves VAMP-2, inhibited eosinophil degranulation (52). This indicates that VAMP-2 has a functional role in eosinophil degranulation. In basopohils only SNAP-23 has been detected (own unpublished results).

T- AND B-LYMPHOCYTES AND DENDRITIC CELLS

Both the lymphocytes and dendritic cells are part of the immune defence of mammals against foreign invaders. Dendritic cells present antigens to the T-cells, which by releasing cytokines attract the other inflammatory cells and thereby cause inflammation. In the inflammatory process, the B-lymphocytes release antibodies. By binding to the effector cells, the antibodies activate these cells (53).

Exocytosis from lymphocytes and dendritic cells may happen through regulated exocytosis. There is circumstantial evidence that some T-cells release preformed cytokines upon stimulation via the T-cell receptor (N. Ødum, personal communications). So far no proteins participating in regulated exocytosis have been identified in these cells.

New insights regarding SNAREs in regulated exocytosis

Interest in the SNARE-hypothesis has been great because it may explain all intracellular vesicular fusion events. Many of the protein–protein interactions postulated by the SNARE-hypothesis have been confirmed over the past few years. However, recent work has questioned some of the postulates of the SNARE-hypothesis. One question concerns the specificity of vesicle traffic. Is it regulated by the SNARE-complex formation alone, or do other regulatory mechanisms exist?

The essential postulate of the original SNARE-hypothesis was that a v-SNARE interacted with two t-SNAREs to determine target specificity in membrane trafficking. However, now it appears that the SNARE-proteins participate in the formation of several different SNARE complexes and therefore are able to pair with more than one set of partners (28,54). Therefore, additional regulatory proteins may contribute to the specificity. In addition, no prevention of vesicle docking was seen in *Drosophila* expressing deficient syntaxin (55). The finding that both t-SNAREs, SNAP-25 and syntaxin, are present on vesicles, has questioned the docking process described by the SNARE-hypothesis even more (56). Recently, calcium was found to induce fusion without formation of the SNARE-complex. When the SNARE-complex was disrupted, vesicles were still able to bind to the plasma membrane and retain full ability for Ca^{2+}-triggered exocytosis (57). Thus several factors other than v- and t-SNAREs determine the specificity of vesicle-target membrane fusion.

Another question of the original SNARE-hypothesis is: when does NSF/SNAP enter the process of vesicle docking and fusion?

According to the original SNARE-hypothesis NSF and SNAP act on the SNARE-complex at the point of vesicle docking and immediately before fusion. However, new data indicate that NSF and SNAP act on SNARE complexes on undocked vesicles. NSF and α-SNAP have been purified from secretory vesicles (58), and it has been shown that they disassemble the SNARE-complex on the vesicles (59). Since NSF/SNAP have been shown to change the conformation of syntaxin (60), it is possible that NSF/SNAP, by binding to the SNARE complex on the vesicles, can modify the conformation of the SNAREs and thereby prepare vesicles for the following round of docking and fusion. In addition, it could be that NSF/SNAP also could prepare t-SNAREs located on the plasma membrane for vesicle docking (60).

The original SNARE-hypothesis can explain many intracellular events, but there are still some unsolved questions. Therefore, the SNARE-complex hypothesis has to be reassessed to give a more clear picture of the molecular machinery controlling regulated exocytosis.

Perspectives

Attention has to be paid to prevention of the inflammatory cascade before it gets established. By preventing the release of soluble mediators from the inflammatory cells, several

toxic effects caused by these cells can be inhibited. Here it is important to understand the SNARE-machinery in inflammatory cells. The proteins implicated in the regulated exocytosis from the inflammatory cells can be a whole new set of promising targets for drugs. Future challenges are to elucidate the actual functional role played by proteins mediating exocytosis in the inflammatory cells. This exciting field of future research will hopefully provide us with new strategies for therapy.

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